

Reduced diet and reduced reproduction both increase lifespan in lubber grasshoppers, perhaps by independent mechanisms

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Summary

Dietary restriction and reduced reproduction both extend lifespan in many animals. Because dietary restriction decreases reproductive output in most species, the long-standing view has been that both extend lifespan via the same fundamental mechanism: fewer nutrients are allocated to reproduction, allowing greater somatic maintenance and storage. We have used grasshoppers to address the linkages among nutrition, reproduction, and longevity. Female grasshoppers offered 70% of an ad libitum diet lived nearly twice as long as females fed ad libitum. Females on the 70% diet did not delay oviposition, reduce lifetime output of vitellin (i.e., egg protein), or reduce levels of hemolymph storage proteins in comparison to controls. In separate experiments, ovariectomy increased survivorship by 30% over sham controls. In contrast to dietary restriction, old ovariectomized females had lower levels of reproductive protein and higher levels of storage protein than controls. These data suggest that dietary restriction and ovariectomy extend lifespan in grasshoppers by different mechanisms.

Introduction

The regulation of lifespan is one of the most fundamental yet least understood aspects of life histories of animals. Nutrition clearly plays an important role in aging because reducing the quality of food available extends lifespan in many species [1]. Understanding how nutrition affects longevity in insects may lead to pest control methods.

Dietary restriction, under eating while avoiding malnutrition, extends lifespan in nearly all species tested, from worms to dogs [1]. Several potential mechanisms by which dietary restriction may extend lifespan have been proposed. These include reduction of insulin / insulin-like peptide signaling [2], activation of the histone deacetylase sir2 [3], and increase in expression of the *pha-4* gene that participates in glucose homeostasis [4](Panowski et al. 2007). These mechanisms are not necessarily mutually exclusive, and other pathways may also be involved. In particular, in most animals, dietary restriction also results in a reduction in fecundity, which is also believed to contribute to longevity [5](Partridge et al. 2005).

At the same time, reducing reproduction directly (e.g., by ovariectomy) extends lifespan in many species [e.g., worms, 6 and 7; mice, 8; fruit flies, 9; reviewed in 5]. Because life-extending dietary restriction also reduces reproduction in most species, the same mechanism is often used to explain both of these routes to longer a lifespan.

The disposable soma (a.k.a., nutrient allocation) hypothesis suggests that life extension occurs by reducing nutrient investment in reproduction and thereby allotting more nutrients to somatic maintenance and storage [10,11]. Most work on the regulation of lifespan is done in organisms such as *Drosophila* that are superb for genetics but challenging for physiology due to their small size. As a result, there has long been convincing circumstantial organismal-level evidence that is consistent with nutrient allocation underlying life extension via both dietary restriction and reduced reproduction, but there are few physiological tests of the hypothesis [e.g., 12]. A recent study rigorously tracked ingested nutrients in fruit flies on dietary restriction. This study found that absolute investment in the soma by flies on dietary

restriction was not greater than absolute investment in the soma by control flies. However, the investment in the soma *relative* to investment in reproduction was greater in flies on dietary restriction than in fully fed controls [13](O'Brien et al. 2008). Hence, the key metric for this hypothesis may be the relative allocation to the soma in comparison to reproduction. In the end, the prediction of the disposable soma hypothesis that nutrient allocation is critical to life extension was supported in flies.

Previous studies on fruit flies and nematode worms have examined the relationship of reduced diet and reduced reproduction. In flies, the *ovo^{Dl}* mutation blocks vitellogenesis and extends lifespan, and subjecting *ovo^{Dl}* mutants to dietary restriction extends lifespan even further. Similarly, flies subjected to X-irradiation to destroy germline cells are long lived, and subjecting X-irradiated flies to dietary restriction extends lifespan still further [14]. Hence, in flies, life extension caused by reduced diet and reduced reproduction are additive, suggesting they extend lifespan by independent mechanisms. Studies in nematode worms show a different pattern. Ablating the germline cells extends lifespan [7], and the *eat-2(as1116)* mutation disrupts pharyngeal pumping to cause dietary restriction and extend lifespan, but combining these two treatments does not increase lifespan further. In contrast to flies, in worms the data suggests that reduced diet and reduced reproduction via germline removal are not additive [15]. Additional studies on other experimental systems may help sort out the general pattern. Notably, none of these studies measured the physiological investments to reproduction or somatic storage within the animals.

We have used lubber grasshoppers (*Romalea microptera*) to address the linkages among nutrition, reproduction, and longevity. Protein is clearly the limiting nutrient for egg production in phytophagous insects [16-18]. In contrast to most vertebrates, insects can store considerable levels of amino acids. This is particularly critical for phytophagous insects on dietary restriction. Amino acids are stored as hexamerins, a conserved family of storage proteins, usually made of six ~75kDa subunits [19,20]. The main depot for proteins in insects is the hemolymph [16]. Hexamerins are produced in the fat body and then secreted into the hemolymph for storage. In lubber grasshoppers, these storage proteins make up 80% of the non-vitellogenin protein throughout the first clutch, so simply measuring total hemolymph protein is a good estimate of protein storage for the animal [21].

The physiological underpinnings of dietary plasticity in production of the first clutch by lubber grasshoppers are well studied [see 22 for a review]. During egg production, storage proteins rise to maximal levels about two weeks before oviposition, and then levels fall steadily until laying. This suggests amino acids are stored in the hemolymph proteins and later used during egg production. Not surprisingly, females offered only ~30% of ad libitum feeding levels produced smaller clutches and later clutches than ad libitum controls [23]. Reduced feeding rates delayed the timing of the peak levels of juvenile hormone (the main gonadotropin) and vitellogenin (the hemolymph precursor to egg yolk-protein). Perhaps most important, lowered feeding also reduced the maximum level of hexameric storage proteins ~40% and delayed the timing of that maximum level [21,24,25].

Reproductive output can be reduced via ovariectomy, yet ovariectomized grasshoppers remain vitellogenic [26]. This distinguishes grasshoppers from mice and fruit flies, two common model organisms for studying aging. Both mice and flies require an ovarian hormone (estrogen and ecdysone respectively) to invest in reproduction [27,28], but lubber grasshoppers do not. Indeed, vitellogenin levels in ovariectomized grasshoppers reach very high levels, because the protein cannot be sequestered into the ovary [26]. Hence,

ovariectomy does not prevent allocation of the limiting nutrient to reproduction, so grasshoppers are a great model for examining the role of the ovary in nutrient allocation and longevity.

Here, we compare feeding rates, survivorship, levels of reproductive proteins, and levels of protein storage underlying life-extending dietary restriction [18] and ovariectomy [29] in grasshoppers. We use these comparisons to address whether dietary restriction and ovariectomy are extending lifespan by the same mechanism of nutrient allocation, or by separate mechanisms.

The disposable soma hypothesis on the effects of reproduction on aging [10,11] allows specific predictions on how feeding rates, levels of reproduction proteins, and levels of protein storage would be affected in phytophagous insects showing enhanced longevity. First, insects with experimentally reduced reproduction may be expected to reduce ingestion; while this outcome is not necessary to support the hypothesis, it would be consistent with dietary restriction and reduced reproduction acting in the same way. Second, and most important, both dietary restriction and reduced reproduction must reduce levels of reproductive protein. Third, both methods could increase levels of protein storage. The disposable soma hypothesis focuses on increased somatic maintenance, not necessarily increased storage. But because protein is the limiting nutrient in insect reproduction [16,17], and insects store hexameric proteins in preparation for major life history events [19,22], a reduction in reproductive protein seems likely to result in an increase in stored protein.

Materials and Methods

For both studies, juvenile grasshoppers were collected, reared in the lab *en masse* at room temperature until adult molt, and then transferred to individual cages to start the experiment. A 14L:10D photoperiod with a corresponding 32°C:24°C thermocycle was used.

Dietary restriction

Romalea microptera from Jacksonville, FL, USA (30°N latitude) were used to test the effects of dietary restriction on grasshopper longevity, levels of reproductive proteins, and protein storage [18]. We compared grasshoppers on an ad libitum diet of Romaine lettuce with grasshoppers offered 70% of that consumed by the ad libitum group (i.e., a dietary restriction group). As the experiment progressed, each day we measured the amount consumed by the ad libitum group, and each week we used these data to adjust the amount offered to the dietary restriction group [18, see Fig. 1 of this paper].

Vitellin in eggs was estimated by collecting laid eggs throughout the study. Insect eggs are typically 50% protein [16], and 90% of this protein is vitellin in grasshoppers (HD Wood and DW Borst, unpublished data). Hence, we determined dry egg masses and multiplied this mass by 50% and then by 90% to estimate vitellin in eggs.

To assess storage protein levels, hemolymph samples were collected every seven days, stored at -20°C until analysis, and tested for total hemolymph protein. Because it is known that the three hexameric storage proteins make up 70% of the total hemolymph protein throughout the first clutch [21], total hemolymph protein values were multiplied by 70% to estimate storage protein levels.

Reduced reproduction by ovariectomy

Grasshoppers from Miami, FL, USA (26°N latitude) were used to test the effects of directly reduced reproduction on longevity, levels of reproductive proteins, and protein storage [29]. We compared ovariectomized and sham operated grasshoppers. Surgeries were performed during the first three days of adulthood as described [26]. Each day, each female was offered 4 g of fresh Romaine lettuce; this offering was rarely completely consumed. From the food remaining the next day, the amount eaten was scored on a 1 – 5 scale.

Reproductive protein was estimated at two points during the study, just after the first clutch for sham females and then at the termination of the study. For ovariectomized females, we measured the vitellogenin concentration in the hemolymph by ELISA [30] and then multiplied this by the typical hemolymph volume of a reproductive female (2.25 ml; S. Li and D.W. Borst, unpublished data). For sham operated females, reproductive protein also accumulates in the eggs, so total reproductive protein was estimated as vitellin in eggs (as for the dietary restriction study) plus vitellogenin in the hemolymph (as for the ovariectomized females).

Total hemolymph protein levels were estimated as in the dietary restriction study, and then vitellogenin level was subtracted from this to estimate the level of hemolymph storage protein [as in 21].

Comparisons of the Jacksonville and Miami populations

These two populations do not differ from each other in reproductive tactics [31]. Nonetheless, there is a high level of population variation in reproductive tactics, physiology, and longevity among other populations of these grasshoppers [23,25,32]. For example, grasshoppers from Athens, GA, USA (34°N latitude) clearly differ from both Jacksonville and Miami grasshoppers in timing of first oviposition and somatic storage after first oviposition [31]. Hence, it is possible that lifespans of the two populations are different. Indeed, this seems probable from the fact that the median age of death for ad libitum grasshoppers (from Jacksonville) was 83 d, while the median age of death for sham operated grasshoppers (from Miami) was 167 d. Because of this, direct comparisons between the two populations may not be valid. To adjust for this, we normalized ages for all presentation of the data, with the median lifespan for the control group set at 100 (e.g., see Fig. 1).

Results

Dietary restriction

For the dietary restriction study, amounts eaten were greatest during the first two weeks measured and then remained somewhat lower until death (Fig. 1A). The dietary restriction group consumed 71% of that eaten by the ad libitum group throughout the course of the study [18]. The amount offered to the dietary restriction group was determined from the amount consumed by the ad libitum group for the previous week. During the third week, there was an abrupt decrease in the amount eaten by the ad libitum group. This created a week in which the dietary restriction group actually consumed more than the ad libitum group. Nonetheless the dietary restriction group ate less throughout the study and had greater longevity than the ad libitum group (Fig. 2A).

Life extending dietary restriction did not greatly affect levels of reproductive proteins (Fig. 3A). Shortly after the median age of first oviposition (normalized age ~ 60), levels of reproductive protein were very similar. During the remainder of the study, reproductive

protein levels seem to increase at similar rates in both the dietary restriction and ad libitum groups. Lifetime fecundity was not altered by dietary restriction [18].

Life extending dietary restriction also did not greatly affect levels of hemolymph storage protein (Fig. 4A). The dietary restriction and ad libitum groups had surprisingly similar levels of storage proteins throughout the study. Levels in both groups were highest in the middle of the first clutch, fell to lower levels around the time of oviposition, and then remained low for the remainder of the study.

Reduced reproduction by ovariectomy

For the ovariectomy study, amounts eaten were similar throughout the study for ovariectomized and sham operated females (Fig. 1B). Despite this, after the median age of death for sham females (i.e., normalized age = 100); ovariectomized females had about 30% higher survivorship [29].

Shortly after the median age of first oviposition (normalized age ~ 30), levels of reproductive proteins were similar in ovariectomized and sham operated females (Fig. 3B). By the termination of the study (normalized age ~ 120), levels of reproductive proteins had decreased in ovariectomized females, whereas levels of reproductive proteins had increased in sham operated females.

In contrast to reproductive proteins, levels of hemolymph storage proteins increased in ovariectomized females from shortly after the first clutch until the median age of death in sham females (i.e., normalized age = 100). In sham operated females, levels of hemolymph storage proteins decreased during the same period (Fig. 4B). In summary, at older ages, ovariectomy decreased levels of reproductive proteins and increased levels of hemolymph storage proteins.

Conclusions

If dietary restriction and ovariectomy both act through nutrient allocation to extend lifespan in grasshoppers, then several predicted outcomes of our experiments would be expected. Females on dietary restriction should decrease levels of reproductive proteins and perhaps increase levels of stored proteins. Similarly, ovariectomized females might reduce feeding rates, and they should decrease levels of reproductive proteins and perhaps increase stored proteins. These predictions were not consistently matched by our data. Dietary restriction extended lifespan in females without altering output of reproductive protein or levels of protein storage in the hemolymph. In contrast, ovariectomy extended lifespan without altering feeding rates, while reducing levels of reproductive proteins in older females (but not younger females) and increasing levels of protein storage in the hemolymph. These data suggest that ovariectomy extends lifespan by shifting nutrient allocation from reproduction to the soma, as predicted by the disposable soma hypothesis. In contrast, dietary restriction seems to extend lifespan by some means independent of nutrient allocation. Hence, both dietary restriction and ovariectomy extend lifespan in female grasshoppers, but may do so in different ways.

Role of diet in increased lifespan upon reduced reproduction

Reduced reproduction may cause reduced feeding and in effect dietary restriction. This has rarely been addressed, because ingestion is not easily measured in fruit flies and nematode worms, the species most often used for lifespan research. Recently, several labs have attempted to quantify ingestion in fruit flies, resulting in a contentious literature [33-37]. This

may have been settled (38,39), but at the very least, measuring consumption in 1 μg fruit flies is arduous. In contrast, ingestion is easy to measure in 5 g grasshoppers.

Surprisingly, life extending ovariectomy did not alter feeding rate in grasshoppers (Fig. 1). This is surprising because another method of reducing reproduction in grasshoppers, namely allatectomy (surgical removal of the gland that makes the primary gonadotropin), reduces feeding by about two-thirds [40]. Hence, analysis of the amount eaten during adulthood does not support the notion that both dietary restriction and reduced reproduction via ovariectomy extend lifespan by the same means.

Allocation of protein to reproduction vs. storage upon dietary restriction or ovariectomy

The disposable soma hypothesis suggests that reproduction and the soma compete for nutrients. If there is such an antagonism, the nutrient that limits reproduction would be competed for most intensely. Protein is the limiting nutrient for reproduction in phytophagous insects. These insects consume large quantities of carbohydrate-rich plant material, so energy is unlikely to be limiting and may not be an appropriate metric when testing hypotheses on resource allocation. Specifically quantifying protein committed to reproduction vs. storage should best speak to whether dietary restriction and ovariectomy are extending lifespan by nutrient allocation.

Dietary restriction in grasshoppers extended lifespan without altering lifetime fecundity or hemolymph protein storage. Our method of limiting diet was to offer 70% of the quantity consumed by ad libitum controls. This is quite different than most studies utilizing dietary restriction, which use diluted food offered ad libitum [41]. Many insects have been shown to exhibit compensatory feeding when offered low quality food offered ad libitum [e.g., 42 for crickets). Because of this, in studies that use diluted food offered ad libitum for studying aging, there must be some additional evidence that nutrition was limited. Some authors suggest that some measure of performance other than lifespan must be measured, such as fecundity [43]. That is, it is only clear that flies have been subjected to dietary restriction if there is a cost observed, often reduced fecundity. This is the reduced reproduction that is seen in most animals on dietary restriction. However, when dietary restriction is administered as a reduced *quantity* of food, no compensatory feeding is possible. Hence, a legitimate outcome of the experiment is enhanced lifespan without an observed cost such as reduced reproduction. It seems likely there must be a cost incurred to extend lifespan, but this need not be observed to verify that the diet was limited, as this is established by the experimental design. Because of this, the observation of dietary restriction without reduced fecundity or storage is reasonable in our studies. Interestingly, the *eat-2(ad1116)* worm mutation inhibits pharyngeal pumping [15], so this likely causes a reduction in diet quantity; when the experiments on the relationship of reduced diet and reduced reproduction were repeated using normally feeding worms on diluted food, the results changed [15]. Together, these studies on grasshoppers and worms illustrate the importance of measuring ingestion when addressing the role of the diet and nutrients in the relationship of reproduction and lifespan.

At the age when intact females grasshoppers lay their first clutch, the amounts of reproductive protein produced are similar in ovariectomized females and sham females, even though the proteins are vitellogenin in the ovariectomized females but mostly vitellin in eggs in the sham females. Ultimately, ovariectomy results in reduced levels of reproductive protein, consistent with predictions of the disposable soma hypothesis. In contrast, females on life extending dietary restriction were able to continue producing clutches during their entire lifespan. Neither age at first oviposition nor lifetime fecundity differed between the dietary

restriction and ad libitum groups [18]. Hence, there is a clear difference in how these two methods of increasing lifespan affect the production of reproductive protein.

The disposable soma hypothesis predicts that the protein saved by reducing reproduction is used for increased somatic maintenance, perhaps as increased storage. Indeed, previous studies on fruit flies have shown greater lipid storage in long-lived phenotypes [12,44]. Because protein is the limiting nutrient for reproduction, storage of amino acids in proteins is likely to be more important than the storage of lipids [18]. Levels of hexameric storage proteins are much higher in old ovariectomized females than in sham females. At the same time, dietary restriction did not change levels of hemolymph storage proteins (Fig. 4). Similar to reproductive protein, there is a clear difference in how these two methods of increasing lifespan affect protein storage.

Future research

Our results to this point on life extension in grasshoppers are consistent with the notion that ovariectomy extends lifespan via some nutrient allocation mechanism, but dietary restriction extends lifespan by some other means. To further test whether these two methods enhance lifespan by the same mechanism we plan a 2 x 2 full factorial test of dietary restriction and ovariectomy on lifespan. That is, we will measure the lifespan of four groups: sham operated & ad libitum diet, sham operated & dietary restricted, ovariectomized & ad libitum diet, and ovariectomized & dietary restricted. The feeding levels for the dietary restricted groups will be 70% of the amount eaten by individuals in the ad libitum group subjected to the same surgery.

A second approach to more rigorously testing whether life extension occurs by nutrient allocation is to feed grasshoppers labelled diets and then measure the amounts of ingested nutrients allocated to reproduction protein vs. somatic and stored protein [45,46]. In pilot experiments, we have shown that grasshoppers can reproduce on diets that differ in ^{13}C levels. These diets consisted of 0.5 g Romaine lettuce (which alone is insufficient for reproduction) and ad libitum artificial diet daily. The artificial diet includes agar, cellulose, and a protein and lipid source. We have identified two dog foods that can be used as the protein source for the diet yet differ in ^{13}C levels. The timing of oviposition and clutch size of grasshoppers on the diet with higher ^{13}C levels was similar to grasshoppers on an ad libitum diet of lettuce, which is known to be a favourable diet. We intend to test whether old ovariectomized females allocate ingested nutrients to protein storage at the expense of protein for reproduction.

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Figure 1. Amounts of Romaine lettuce eaten by female lubber grasshoppers throughout adulthood, adjusted for body mass at age 4 days. Lettuce was always offered fresh. Values for the dietary restriction study (Panel A) were determined by collecting, drying and weighing uneaten lettuce. Values for the ovariectomy study (Panel B) were estimated by scoring the amount of uneaten food. Ovariectomized and sham operated females consumed similar amounts throughout the experiment. Because the two studies used grasshoppers from different populations, and populations within this species are known to differ in reproductive tactics, values between the two experiments may not be comparable. To make the ages across the two studies more comparable, normalized ages were calculated as $((\text{age in days} / \text{median age of death of the control group in days}) * 100)$. The ad libitum and sham operated groups were the control groups for these calculations.

Figure 2. Survivorship of female lubber grasshoppers throughout adulthood. Panel A shows the result of an experiment on effects of diet quantity on lifespan, and Panel B shows the results of an experiment on ovarian presence on lifespan. Both dietary restriction and ovariectomy extend lifespan. Because the two studies used grasshoppers from different populations, and populations within this species are known to differ in reproductive tactics, values between the two experiments may not be comparable. To make the ages across the two studies more comparable, normalized ages were calculated as $((\text{age in days} / \text{median age of death of the control group in days}) * 100)$. The ad libitum and sham operated groups were the control groups for these calculations.

Figure 3. Reproductive protein levels in female lubber grasshoppers at different moments during adulthood, adjusted for body size at age 4 days. Vitellin in eggs for the dietary restriction study (Panel A) was determined by collecting, drying and weighing laid eggs. Life extending dietary restriction had no significant effect on reproductive output. Total reproductive protein for the ovariectomy study (Panel B) was determined in two different ways. Ovariectomized females cannot lay eggs, so reproductive proteins for this group were estimated by measuring hemolymph vitellogenin concentrations by ELISA and multiplying by the typical hemolymph volume of a female (i.e., 2.25 ml). Sham operated females can lay eggs and make vitellogenin, so total reproductive protein for this group was estimated as vitellin in eggs plus vitellogenin in the hemolymph. Young ovariectomized females produced normal levels of reproductive protein, but older ovariectomized females showed decreased levels of reproductive proteins. Because the two studies used grasshoppers from different populations, and populations within this species are known to differ in reproductive tactics, values between the two experiments may not be comparable. To make the ages across the two studies more comparable, normalized ages were calculated as $((\text{age in days} / \text{median age of death of the control group in days}) * 100)$. The ad libitum and sham operated groups were the control groups for these calculations.

Figure 4. Hemolymph storage protein levels in female lubber grasshoppers through adulthood. Values for the dietary restriction study (Panel A) were determined by measuring total hemolymph protein and then multiplying by 70%. Previous studies showed that three hemolymph hexamerins make up 70% of the total hemolymph protein (Hatle et al. 2001). Values for the ovariectomy study were determined by measuring total hemolymph protein and subtracting vitellogenin (determined by ELISA). Previous studies suggest that vitellogenin is ~10% of total hemolymph protein throughout the first clutch. Further, because the two studies used grasshoppers from different populations, and populations within this species are known to differ in reproductive tactics, values between the two experiments may not be comparable. To make the ages across the two studies more comparable, normalized

ages were calculated as $((\text{age in days} / \text{median age of death of the control group in days}) * 100)$. The ad libitum and sham operated groups were the control groups for these calculations.







